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REGULAR ARTICLE

Embryoid body attachment to reconstituted basement membrane induces a genetic program of epithelial differentiation via jun N-terminal kinase signaling

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Abstract Embryonic stem (ES) cells are derived from early stage mammalian embryos and have broad developmental potential. These cells can be manipulated experimentally to generate cells of multiple tissue types which could be important in treating human diseases. The ability to produce relevant amounts of these differentiated cell populations creates the basis for clinical interventions in tissue regeneration and repair. Understanding how embryonic stem cells differentiate also can reveal important insights into cell biology. A previously reported mouse embryonic stem cell model demonstrated that differentiated epithelial cells migrated out of embryoid bodies attached to reconstituted basement membrane. We used genomic technology to profile ES cell populations in order to understand the molecular mechanisms leading to epithelial differentiation. Cells with characteristics of cultured epithelium migrated from embryoid bodies attached to reconstituted basement membrane. However, cells that comprised embryoid bodies also rapidly lost ES cell-specific gene expression and expressed proteins characteristic of stratified epithelia within hours of attachment to basement membrane. Gene expression profiling of sorted cell populations revealed upregulation of the BMP/TGFβ signaling pathway, which was not sufficient for epithelial differentiation in the absence of basement membrane attachment. Activation of c-jun N-terminal kinase 1 (JNK1) and increased expression of Jun family transcription factors was observed during epithelial differentiation of ES cells. Inhibition of JNK signaling completely blocked epithelial differentiation in this model, revealing a key mechanism by which ES cells adopt epithelial characteristics via basement membrane attachment.

Introduction

Embryonic stem (ES) cells are derived from early stage mammalian embryos and have broad developmental potential. These cells can be manipulated experimentally to generate cells of multiple tissue types which could be important in treating human diseases. The ability to produce relevant amounts of these differentiated cell populations creates the basis for clinical interventions in tissue regeneration and repair (for review, see (Murry & Keller, 2008)). Under appropriate conditions, ES cells have been shown to differentiate into dopamine neurons that functioned in animal models of Parkinson's disease (Kim et al., 2002). ES cell differentiation into motor neurons for use in spinal cord injury has also been studied (Wichterle et al., 2002). ES cells have been differentiated into insulin-secreting pancreatic islet cells for potential treatment of diabetes mellitus (Lumelsky et al., 2001). Models used to differentiate ES cells include forming three-dimensional clusters known as

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embryoid bodies and culture on extracellular matrix or supportive stroma.

A previously characterized mouse ES cell model demonstrated that epithelial cells grew out of embryoid bodies attached to reconstituted basement membrane (Turksen & Troy, 1998; Troy & Turksen, 2006; Troy & Turksen, 2005). In human ES cells, the outgrowth population expressed keratin proteins found in stratified squamous epithelia and decreased ES cell markers (Green et al., 2003; Aberdam et al., 2008). ES cells have also been used in organotypic cultures to generate epidermal equivalents in vitro (Hewitt et al., 2009; Metallo et al., 2010; Coraux et al., 2003). Integrin, growth factor, and transcriptional signaling were required for ES cell differentiation into stratified epithelial cells (Bagutti et al., 1996; Bagutti et al., 2001; Medawar et al., 2008). Understanding how ES cells differentiate into epithelial cells can reveal important insights into cell biology. We used genomic technology to profile differentiating ES cell populations in order to understand the molecular mechanisms leading to the development of epithelial cells. In this model, cells with characteristics of stratified squamous epithelium migrated from embryoid bodies attached to reconstituted basement membrane. Additionally, some cells that comprised the embryoid bodies rapidly lost ES cell-specific gene expression and expressed proteins characteristic of stratified epithelia within hours of attachment to reconstituted basement membrane. Expression of growth factors in the BMP/TGFβ family were upregulated during this process but were not sufficient to induce epithelial differentiation in the absence of basement membrane attachment. Instead, our results demonstrate that JNK1 signaling is critical for the development of epithelia from embryonic stem cells.

Results

A typical ES cell colony grown on mitomycin C-treated STO mouse embryo fibroblasts is shown by phase-contrast microscopy in Fig. 1A. Note that the ES cells are small, tightly packed, and have smooth outlines. Embryoid bodies (EBs) are produced by culturing the ES cells in suspension culture for several days in the absence of leukemia inhibitory factor (LIF). A typical EB growing in suspension culture is shown in Fig. 1B. When these EBs were plated on reconstituted basement membrane, outgrowth of an epithelioid monolayer was observed (3-day culture is shown in Fig. 1C). To determine if these cells expressed epithelial-specific proteins, we performed immunocytochemistry using pan cytokeratin antibody on 3-day cultures. As shown in Fig. 1D, both the epithelial monolayers and the EBs were intensely immunopositive using the anti-pan-cytokeratin antibody on 3-day cultures. To examine early stages of differentiation in this model, we examined EBs attached to reconstituted basement membrane at 24 h by immunofluorescence. An attached EB incubated with control IgG and counterstained with DAPI is shown in Fig. 1E. We detected specific protein markers of stratified epithelial differentiation in the attached EBs, including keratin 18 (Fig. 1F), keratin 19 (Fig. 1G), keratin 5 (Fig. 1H), keratin 1 (Fig. 1I), and filaggrin (Fig. 1J). To determine the percentage of positive cells for each of these epithelial markers, we dissociated 24 h attached EBs to single cells and performed immunofluorescent flow cytometry with individual antibodies. As shown in Fig. 1K, pan keratin antibody labeled 98% of attached EB cells. Anti-keratin 18 antibody labeled 97% of attached EB cells while anti-keratin 19, keratin 5, keratin 1, and filaggrin labeled 61, 38, 12, and 5%, respectively. These results indicate that cells within the EB express markers of simple and stratified epithelial differentiation in this model system.

To determine which markers of epithelial differentiation were expressed by EBs, Matrigel-attached EBs, and monolayer epithelial cells from EBs, we performed real-time RT-PCR using a panel of primers to detect simple and stratified epithelial gene expression. As shown in Fig. 2A markers of simple epithelial differentiation such keratin 8 and keratin 18 (K8/K18) were highly expressed in Matrigel-attached EBs (11-fold induction, $P<0.001$) and the monolayer epithelial outgrowth (9-fold induction, $P<0.001$), but only at very low levels in EBs grown in suspension culture. The stratified epithelial marker keratin 19 (K19) was also expressed in Matrigel-attached EBs (20-fold induction, $P<0.0002$) and the monolayer cells but not in EBs grown in suspension. We also examined expression of additional markers of stratified epithelial differentiation including keratin 5 (K5), keratin 14 (K14), keratin 1 (K1), keratin 10 (K10), involucrin (inv), and filaggrin (flg). As shown in Fig. 2B, K5 expression was induced 3-fold during epithelial differentiation ($P<0.02$). K14 expression was induced 6-fold ($P<0.05$) in the monolayer epithelial outgrowth cells when compared to EBs. K1 expression was induced 4-fold ($P<0.01$) in both Matrigel-attached EBs and the epithelial outgrowth cells compared to EBs grown in suspension culture. Involucrin and filaggrin expression was barely detectable in EBs but was readily detected in differentiated cells. We also examined expression of K18, K19, K14, K1, and filaggrin proteins by Western blot in EBs, attached EBs, and the epithelial monolayer outgrowth. As shown in Fig. 2C, these proteins were largely undetectable in EBs; however, attachment to reconstituted basement membrane strongly induced expression of K18, K19, and K14 in addition to K1 and filaggrin levels. These results demonstrate that epithelial cells derived from EBs attached to Matrigel express multiple markers of simple and stratified epithelial differentiation.

Our mRNA and protein expression data indicate that cells within the EBs attached to Matrigel undergo epithelial differentiation. To begin to determine the genetic changes responsible for the differentiation of stratified epithelial cells from EBs, we performed gene expression profiling on EBs, EBs attached to reconstituted basement membrane, and the epithelial cell outgrowth of EBs (Fig. 2D). Hierarchical clustering analysis of gene expression profiles between EBs and basement membrane-attached EBs revealed 710 differentially expressed genes. A second comparison of genes expressed by EBs and the stratified epithelial cell population showed 734 differentially expressed genes. However, comparison of the basement membrane-attached EBs to their epithelial cell progeny showed only 167 differentially expressed genes. These results suggested that basement membrane-attached EBs were highly related to the epithelial outgrowth cells.

The genes responsible for the transition from ES to epithelial cells in this system are largely uncharacterized. Gene expression changes between EBs and basement

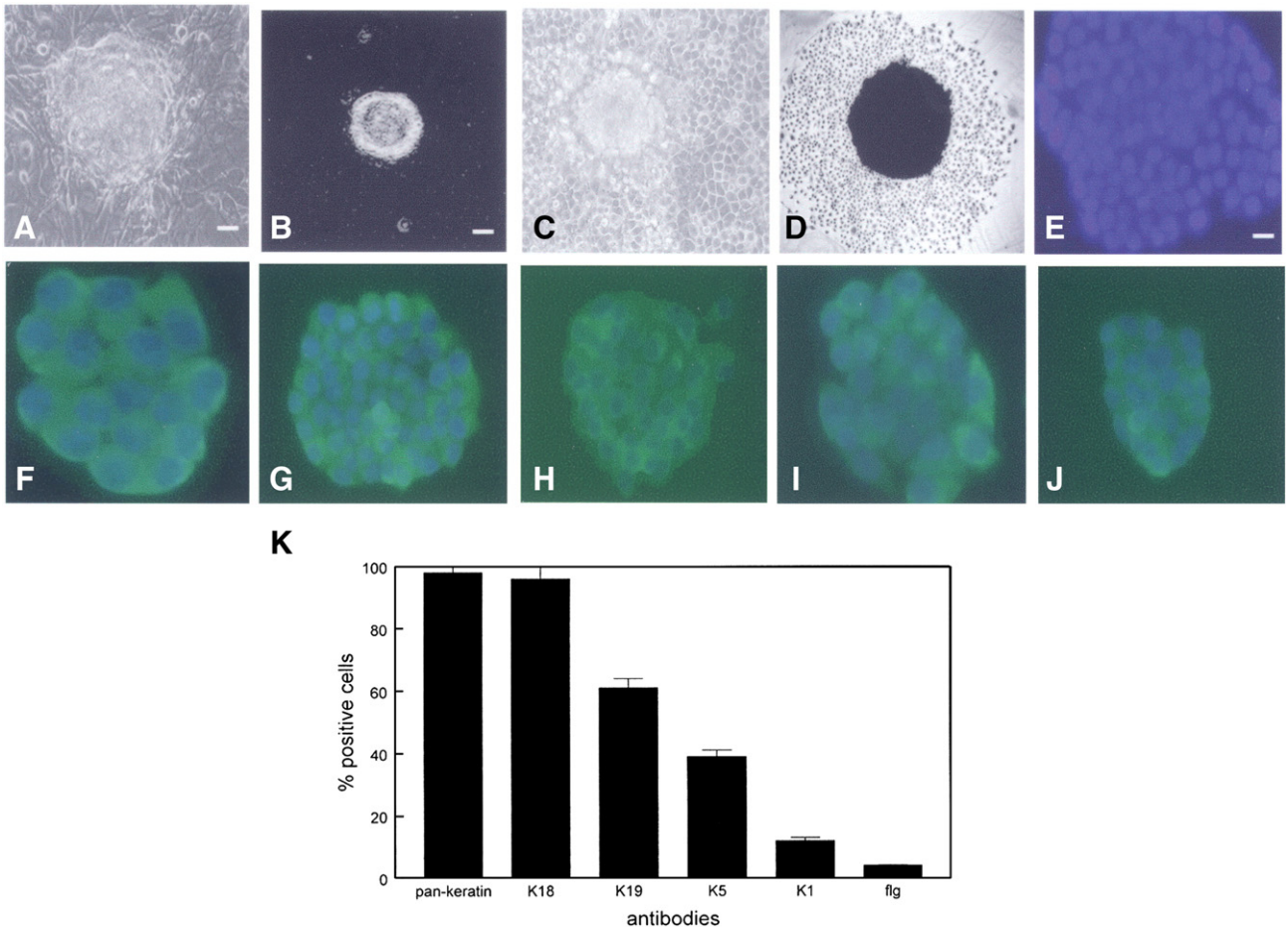
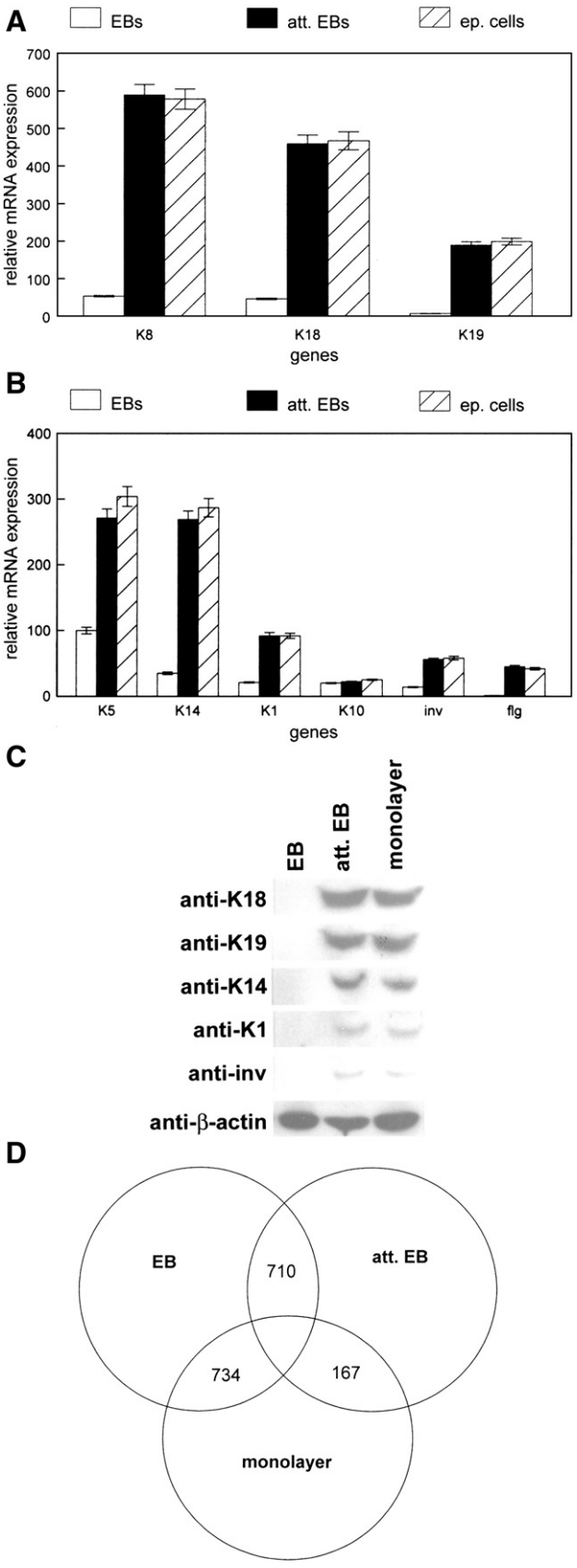


Figure 1 Mouse embryonic stem cells differentiate into stratified squamous epithelial cells when plated on reconstituted basement membrane. (A) Phase-contrast photomicrograph of a mouse embryonic stem cell colony (center) growing on mitomycin-treated STO mouse embryo fibroblasts (background). Scale bar = 20 μm. (B) Phase-contrast photomicrograph of an embryoid body (EB) formed from ES cells after 2 days in suspension culture. Scale bar = 50 μm. (C) Phase-contrast photomicrograph of stratified epithelial cells migrating out of embryoid body (center) after 3 days. (D) Bright-field photomicrograph showing intense pan-cytokeratin immunocytochemical staining of fixed stratified epithelial cells and associated embryoid body. Scale for (C, D) is same as (A). (E) Immunofluorescence photomicrograph of an EB attached to Matrigel for 24 h followed by fixation and incubation with control IgG (no staining) and counterstained with DAPI (blue). Scale bar for immunofluorescent photomicrographs = 20 μm. (F) Immunofluorescence photomicrograph of EB attached to Matrigel for 24 h stained with anti-keratin 18 antibody (FITC, green) and counterstained with DAPI (blue). (G) Immunofluorescence photomicrograph of EB attached to Matrigel for 24 h stained with anti-keratin 19 antibody (FITC, green) and counterstained with DAPI (blue). (H) Immunofluorescence photomicrograph of EB attached to Matrigel for 24 h stained with anti-keratin 5 antibody (FITC, green) and counterstained with DAPI (blue). (I) Immunofluorescence photomicrograph of EB attached to Matrigel for 24 h stained with anti-keratin 1 antibody (FITC, green) and counterstained with DAPI (blue). (J) Immunofluorescence photomicrograph of EB attached to Matrigel for 24 h stained with anti-filaggrin antibody (FITC, green) and counterstained with DAPI (blue). (K) EBs attached to Matrigel were dissociated into single cells, fixed, permeabilized, and incubated with pan-keratin, K18, K19, K5, K1, or filaggrin antibodies followed by FITC-conjugated secondary antibody and sorted by flow cytometry to determine percentage of cells expressing these proteins. Error bars indicate SEM of three independent experiments.

membrane-attached EBs were significant for downregulation of a number of transcripts expressed by ES cells or known to be involved in early embryonic development (Table 1). Genes regulating early embryonic development were significantly downregulated in this model. Expression of the TGFβsuperfamily member growth differentiation factor 3 which is expressed in human embryonal carcinoma cells and downregulated upon differentiation (Metallo et al., 2010) was inhibited. Expression of the ES cell homeobox gene Nanog was downregulated in EBs attached to reconstituted basement membrane. Expression of undifferentiated embryonic cell transcription factor 1, which is expressed in ES and embryonal carcinoma cells as well as germ line tissues (Coraux et al., 2003), was downregulated. Expression of G1/S cyclins D1 and E were also significantly inhibited. We noted upregulation of the transcription factor JunD in EBs attached to Matrigel. Despite Matrigel attachment, expressions of basement membrane components laminin B1 and type IV procollagen α1 chain were significantly upregulated in these EBs. We did not detect expression of the endodermal marker



GATA6, mesodermal marker Brachyury, or ectodermal marker Pax6. These results suggest that while ES cell markers are present in EBs, their mRNA levels are significantly downregulated by basement membrane-induced differentiation which coincides with the onset of epithelial-specific gene expression.

We also compared changes in gene expression patterns between EBs cultured in suspension and the stratified epithelial progeny of EBs attached to reconstituted basement membrane. Those changes in gene expression with the most significant *P* values are shown in Table 2. A number of the same stem cell and embryonic development genes that were downregulated in basement membrane-attached EBs were also decreased in the epithelial cells. The G1/S phase cyclins E and D1 were also downregulated in epithelial cells. Expression of a number of epithelial-specific markers was upregulated in the epithelial cells compared to EBs grown in suspension. In the epithelial outgrowth population we observed increased expression of stratified squamous differentiation markers (13). Another member of the Jun transcription factor family c-jun was upregulated in epithelial outgrowth cells. Increased expression of extracellular matrix mRNAs was also noted. Expression of a number of growth factors particularly in the BMP/TGFβ signaling pathway was increased in epithelial cells. These results demonstrate that attachment-mediated differentiation of stratified squamous epithelial cells from EBs was associated with downregulation of stem cell markers, increased expression of keratinocyte-specific genes, and increased BMP/TGFβ signaling.

We also compared gene expression changes between EBs attached to reconstituted basement membrane and stratified squamous epithelial outgrowth of these EBs. Gene expression analysis showed that these two groups were highly related when compared to EBs cultured in suspension (see Fig. 2D). Those changes in gene expression with the most significant *P* values are shown in Table 3. Markers of

Figure 2 Multiple markers of epithelial differentiation are expressed in EBs attached to Matrigel. (A) Expression of the simple epithelial keratins 8 and 18 and stratified epithelial keratin 19 in EBs, EBs attached to Matrigel (att. EBs), and the EB epithelial outgrowth (ep. cells) was determined by real-time RT-PCR. (B) Expression of stratified epithelial differentiation markers keratin 5, keratin 14, keratin 1, keratin 10, involucrin, and filaggrin in EBs, EBs attached to Matrigel (att. EBs), and the EB epithelial outgrowth (ep. cells) was determined by real-time RT-PCR. (C) Expression of stratified epithelial differentiation markers keratin 18, 19, keratin 14, keratin 1, and filaggrin in EBs, EBs attached to Matrigel (att. EBs), and the EB epithelial outgrowth (monolayer) was determined by Western blot. (D) The relationships among differentially expressed genes in EBs, EBs attached to Matrigel (att. EB), and epithelial cells (monolayer) that migrated out of attached EBs are shown by hierarchical clustering following microarray analysis. A Venn diagram generated from the average linkage hierarchical clustering of gene expression patterns from all experiments is given. EBs attached to reconstituted basement membrane and their epithelial outgrowth cells were more highly related based on gene expression profiles than either population was to the original EBs grown in suspension culture.

Table 1 Gene expression profiling of EBs and basement membrane-attached EBs

Accession	Symbol	Gene name	Fold change
NM_007423	Afp	Alpha fetoprotein	360
NM_009695	Apoc2	Apolipoprotein C2	165
NM_009692	Apoa1	Apoplipoprotein A1	128
U63146	Rbp4	Retinol binding protein 4, plasma	50
BI110565	Postn	Periostin, osteoblast-specific factor	46
AV152953	Ttr	Transthyretin	42
AK008641	Gkn1	Gastrokine 1	30
BM236111	Lpp	LIM domain containing preferred translocation partner in lipoma	23
AI385532	Thbs1	Thrombospondin1	22
BC028826	AI788959	Expressed sequence AI788959	20
AK011118	Fgb	Fibrinogen	20
BB533736	Cyr61	Cysteine rich protein 61	18
NM_008471	Krt1-19	Keratin 19	18
J03458.1	Flg	Filaggrin	18
NM_008879	Lcp1	Lymphocyte cytosolic protein 1	15
NM_010217	Ctgf	Connective tissue growth factor	15
NM_008161	Gpx3	Glutathione peroxidase 3	14
AK009873	Car12	Carbonic anhydrase 12	14
NM_033314	Slco2a1	Solute carrier organic anion transporter family, member 2a1	14
NM_009258	Spink3	Serine protease inhibitor	11
D88690	Flt1	FMS like tyrosine kinase 1	11
U65091	Cited1	CBP/p300 interacting transactivator	11
NM_031170	Krt2-8	Keratin 8	11
NM_010659.1	Krt1-1	Keratin 1	11
NM_021339	Cdon	Cell adhesion molecule related/downregulated by oncogenes	10
NM_008010	Fgfr3	Fibroblast growth factor receptor 3	9
AW322280	Krt2-8	Keratin 8	9
NM_010664	Krt1-18	Keratin 18	7
BF158638	Col4a1	Type IV collagen, alpha chain	5
NM_010284	Ghr	Growth hormone receptor	5
BB151715	Ets1	E26 avian leukemia oncogene 1, 5′ domain	4
BG970109	Lamb1-1	Laminin B1	4
NM_010592	Jund1	Jun proto-oncogene related gene d1	3
X65506.1	Krt1-5	Keratin 5	3
BG873440	Fgfr2	Fibroblast growth factor receptor 2	2
U42190	msh6	mutS homolog 6 (E. coli)	-3
NM_007631	Ccnd1	Cyclin D	-4
BB293079	Ccne1	Cyclin E	-5
NM_007544	Bid	BH3 interacting domain death agonist	-6
NM_008108	Gdf3	Growth differentiation factor 3	-8
NM_009482	Utf1	Undifferentiated transcription factor 1	-8
AK010332	Nanog	Nanog homeobox	-8
U31967	Sox2	SRY-box containing gene 2	-9
AW556396	Slc14a1	Solute carrier family 14 (urea transporter), member 1	-10
BB709552	Fgf4	Fibroblast growth factor 4	-12
BQ173923	Cbl	Cordon bleu	-15
BB292776	Eef1d	Eukaryotic translation EF 1 delta	-18
NM_010094	Leftb	Left right determination factor B	-21
NM_011934	Esrrb	Estrogen related receptor beta	-23
NM_010816	Morc	Micro-orchidia	-25
BB067210	Dppa2	Developmental pluripotency associated 2	-26
AF067062	—	Similar to 2 cell stage variable group member 1	-34
NM_009292	Stra8	stimulated by retinoic acid gene 8	-35
NM_025721	Spesp1	Sperm equatorial segment protein 1	-36

Table 2 Gene expression profiling of EBs and stratified squamous epithelial outgrowth

Accession	Symbol	Gene name	Fold change
BQ128572	–	60 S ribosomal protein L3	329
BC019382	Pip5k1b	phosphatidyl inositol 4 phosphate 5 kinase	200
BQ126025	–	60 S ribosomal protein L21	175
NM_007423	Afp	Alpha fetoprotein	158
BM197200	–	Elongation initiation factor 4 1a	114
BQ126463	–	40 S ribosomal protein S2	86
NM_008341	Igfbp1	Insulin like growth factor binding protein 1	80
NM_008471	Krt1-19	Keratin 19	25
NM_008412.1	Inv	involucrin	25
U08020	Col1a1	Type I collagen	23
BF144658	Tgfb2	Transforming growth factor beta 2	20
BB040443	Snai2	Snail homolog 2	18
J03458.1	Flg	Filaggrin	18
AV239587	Bmp2	Bone morphogenetic protein 2	17
BC010337	Krt2-7	Keratin 7	15
BB533736	Cyr61	Cysteine rich protein 61	14
NM_010664	Krt1-18	Keratin 18	12
NM_009258	Spink3	Serine protease inhibitor 3	11
NM_010514	Igf2	Insulin like growth factor 2	11
NM_010659.1	Krt1-1	Keratin 1	11
AW322280	Krt2-8	Keratin 8	10
BB550124	Tgm2	Transglutaminase 2	9
NM_007559	Bmp8b	Bone morphogenetic protein 8b	9
AI507307	Sbsn	Suprabasin	9
AV239646	Gjb2	Gap junction membrane channel protein beta 2	9
NM_013601	Msx2	homeo box, msh-like 2	8
U91905	Frzb	Frizzled related protein	8
X65506.1	Krt1-5	Keratin 5	8
NM_009829	Ccnd2	Cyclin D2	7
AA499047	Pdgfrb	Platelet derived growth factor receptor	7
BE197934	Krt1-14	Keratin 14	7
NM_010591	jun	Jun oncogene	6
BM250666	Col4a5	Type IV collagen	5
BG066605	Lamc1	Laminin gamma 1	4
BB151715	Ets1	E26 avian leukemia oncogene 1, 5′ domain	4
BI248947	Cald1	Caldesmon	4
BC022107	Cdh2	Cadherin 2	4
BC021592	Pla2g12b	Phospholipase A2	4
AK011935	Smarca2	SWI/SNF related matrix associated regulator of chromatin	4
AW987375	Ptpn21	Protein tyrosine phosphatase, non-receptor type 21	4
X06340	Cdh3	Cadherin 3	3
BB454540	Marcks	Myristoylated alanine rich protein kinase C substrate	3
NM_010580	Itgb5	Integrin beta 5	3
AV327574	Adam10	A disintegrin and metalloproteinase domain 10	3
NM_021329	Rangnrf	Ran guanine nucleotide release factor	-3
NM_007631	Ccnd1	cyclin D1	-4
NM_008602	Miz1	Msx interacting zinc finger	-4
BB293079	Ccne1	cyclin E1	-5
AK010332	Nanog	Nanog homeobox	-8
NM_009482	Utf1	Undifferentiated embryonic cell transcription factor 1	-8
BB709552	Fgf4	Fibroblast growth factor 4	-12
BQ173923	Cbl	Cordon bleu	-15
NM_010094	Leftb	Left right determination factor B	-21
	Morc	Micro-orchidia	-25
BB067210	Dppa2	Developmental pluripotency associated 2	-26
AF067062	–	Clone L4 variable group of 2 cell stage gene family	-34

Table 3 Gene expression profiling of basement membrane-attached EBs and stratified epithelial outgrowth

Accession	Symbol	Gene name	Fold change
BQ126463	–	40 S ribosomal protein S2	86
NM_009692	Apoa1	apolipoprotein A1	78
U63146	Rbp4	retinol binding protein 4	50
AK009959	Ankrd1	ankyrin repeat domain 1	42
BB533736	Cyr61	cysteine rich protein 61	34
AV026617	Fos	FBJ osteosarcoma gene	29
NM_008485	–	laminin gamma 2	25
BM246754	Arhgef6	rac/cdc42 guanine nucleotide exchange factor 6	24
BQ127142	Acp1	acid phosphatase 1	23
AF425083	–	serpin b9g	21
AK01111	Fgb	fibrinogen	19
AI385532	Thbs1	thrombospondin	18
BE650380	Car7	carbonic anhydrase 7	14
NM_133736	Rgs5	regulator of G protein signaling 5	14
AF425083	serpinb9e	serine (or cysteine) proteinase inhibitor, clade B, member 9e	14
NM_007559	Bmp8b	bone morphogenetic protein 8b	9
AV239646	Gjb2	gap junction membrane channel protein beta 2	9
BF144658	Tgfb2	transforming growth factor beta 2	8
AI507307	Sbsn	suprabasin	8
BE852181	Rnd1	Rho family GTPase 1	7
NM_009866	Cdh11	Cadherin 11	6
BC022107	Cdh2	Cadherin 2	4
NM_008955	Psx1	Placenta-specific homeobox 1	4
BI248947	Cald1	Caldesmon 1	3
X06340	Cdh3	Cadherin 3	3
NM_013599	Mmp9	matrix metalloproteinase 9	3
AK005063	Rap1ga1	Rap1, GTPase activating protein 1	3
BB768208	Sgk3	Serum glucocorticoid regulated kinase 3	3
BB454540	Marcks	Myristoylated alanine rich protein kinase C substrate	3
AW546010	Gli3	GLI-Kruppel family member GLI3	3

stratified squamous epithelium were still upregulated in this comparison. Additionally, expression of ligands in the BMP/TGFβ signaling pathway again was increased. These data indicate that EBs attached to basement membrane undergo a shift to stratified epithelial-specific gene expression that is not restricted to outgrowth cells.

Matrigel is composed of type IV collagen in addition to other extracellular matrix molecules such as laminin and fibronectin. To determine which Matrigel components were primarily responsible for inducing epithelial differentiation, we plated EBs onto type IV collagen, laminin, fibronectin, or plastic and monitored production of epithelial cells. As shown in Fig. 3A, type IV collagen was nearly as efficient as Matrigel at inducing expression of the epithelial keratins K8, K18, and K19. Laminin and fibronectin were no more efficient than plastic at inducing keratin expression, although low level expression was detected in all EB cultures. Given the marked upregulation of BMP and TGFβ ligands which correlated with differentiation of EBs into stratified epithelium, we hypothesized that attachment of constituent cells to the reconstituted basement membrane may result in paracrine growth factor signaling which then induces epithelial differentiation of the EB. To begin to test this hypothesis, we confirmed induction of BMP2 and TGFβ2 mRNA expression in EBs, basement membrane-attached EBs, and stratified squamous epithelial cells by RT-PCR (Fig. 3B). TGFβ2 mRNA expression was nearly undetectable in EBs grown in suspension culture but was induced 20-fold in the stratified squamous epithelial outgrowth of basement membrane-attached EBs. BMP2 mRNA expression was approximately 3-fold higher than that of TGFβ2 in EBs cultured in suspension. BMP2 expression also was induced nearly 20-fold during EB differentiation induced by attachment to reconstituted basement membrane. To determine if these factors could induce epithelial differentiation of EBs in the absence of attachment to reconstituted basement membrane, we treated EBs cultured in suspension for up to 4 days with BMP2 and TGFβ2. Recombinant BMP8b was not available for these experiments and its expression was not associated with stratified epithelial phenotypes in null mutant mice. We added the factors in proportion to their highest relative mRNA levels as described under Materials and methods prior to RNA extraction and real-time RT-PCR analysis. As shown in Fig. 3C, no single factor nor combination of factors was able to induce keratin gene expression in EBs in the absence of attachment to reconstituted basement membrane. To control for the absence of growth factors, we cultured EBs in suspension with reduced serum (2%). Surprisingly, EBs cultured in suspension in 2% serum expressed high levels of K8, K18, and K19, similar to those observed in EBs cultured on Matrigel. These results indicate that reduced serum rather than specific growth factors expressed during differentiation was sufficient to induce epithelial differentiation in EBs in the absence of basement membrane attachment.

Given that serum withdrawal can induce stress-activated protein kinase pathways such as Jun N-terminal kinase (JNK1; 15) and that downstream transcription factor targets of this kinase (c-jun, JunD) are upregulated during epithelial differentiation of EBs, we hypothesized that this genetic program may be initiated by JNK1 activation. To test this hypothesis, we examined activation of JNK1 and the extracellular signal regulated kinase ERK1 by phosphorylation in EBs at different stages of differentiation. As shown by Western blot in Fig. 4, Matrigel attachment produced 8-fold induction of activated JNK1 while activated ERK1 expression decreased by 50%. Reduced serum produced 7-fold induction of activated JNK1 in unattached EBs compared to those cultured in 15% serum. Activated ERK1 expression was reduced to undetectable levels by reduced serum. To verify the specificity of these observations we treated EB cultures with the JNK selective pharmacologic inhibitor SP600125 or the upstream ERK inhibitory drug PD98059. JNK1 and ERK1

activation was completely abrogated by SP600125 and PD98059 treatment, respectively. As a positive control we added the BMP2/TGFβ2 growth factor combination to attached EB cultures and monitored JNK1 and ERK activation. Activated ERK1 expression increased by 2-fold consistent with its role in growth factor signaling. JNK1 was also activated by the growth factor combination. Expressions of total JNK1 and ERK1 proteins were not significantly affected by these treatments. These results indicate that a high ratio of JNK1/ERK1 activation correlates with epithelial differentiation of EBs under both Matrigel-attached and -reduced serum culture conditions.

To begin to determine which intracellular signaling pathways were involved in Matrigel-induced epithelial differentiation, EB cultures were treated individually with specific inhibitors. As shown in Fig. 5A, treatment with PD98059 (MEK inhibitor), LY294002 (PI3K inhibitor), U73122 (PLCγ inhibitor), SB203580 (p38 inhibitor), or TDZD8 (GSK3 inhibitor) had no effect on epithelial differentiation as assessed by expression of multiple keratin genes. However treatment with the JNK1 inhibitor SP600125 completely blocked epithelial differentiation and keratin expression. Inhibition of CaM kinase, Akt, or ras also had no effect on stratified squamous differentiation and inhibition of PKC or NF-κB-induced apoptosis in EB cells (data not shown), indicating that these latter two pathways may provide important survival signals for ES cells. To further demonstrate the requirement of JNK1 signaling for epithelial differentiation of EBs, we inhibited expression of the kinase using siRNA. As shown in Fig. 5B, transfection of EBs with JNK1 siRNA inhibited expression of the kinase by >90%. JNK1 inhibition reduced expression of K8, K18, and K19 by 90% in Matrigel-attached EBs, indicating that JNK signaling is primarily responsible for initiating epithelial differentiation in these cultures. Transfection with a control siRNA had essentially no effect on JNK1 or keratin expression. We also examined changes in the cellular morphology of the monolayer outgrowth from EB cultures treated with SP600125. As shown in Fig. 5C, JNK1 inhibition completely blocked formation of epithelial cells (left panel). The monolayer outgrowth in these cultures consisted of spindle-shaped fibroblastoid cells with poorly developed intercellular junctions. The control monolayer outgrowth cells shown in the right panel of Fig. 5C possess the characteristic rounded cobblestone appearance and well-developed intercellular junctions routinely observed in stratified epithelia. Keratin and involucrin gene expression was completely inhibited in SP600125-treated EB cultures (Fig. 5D). These data indicate that JNK1 signaling is critically important to the epithelial differentiation of Matrigel-attached EBs.

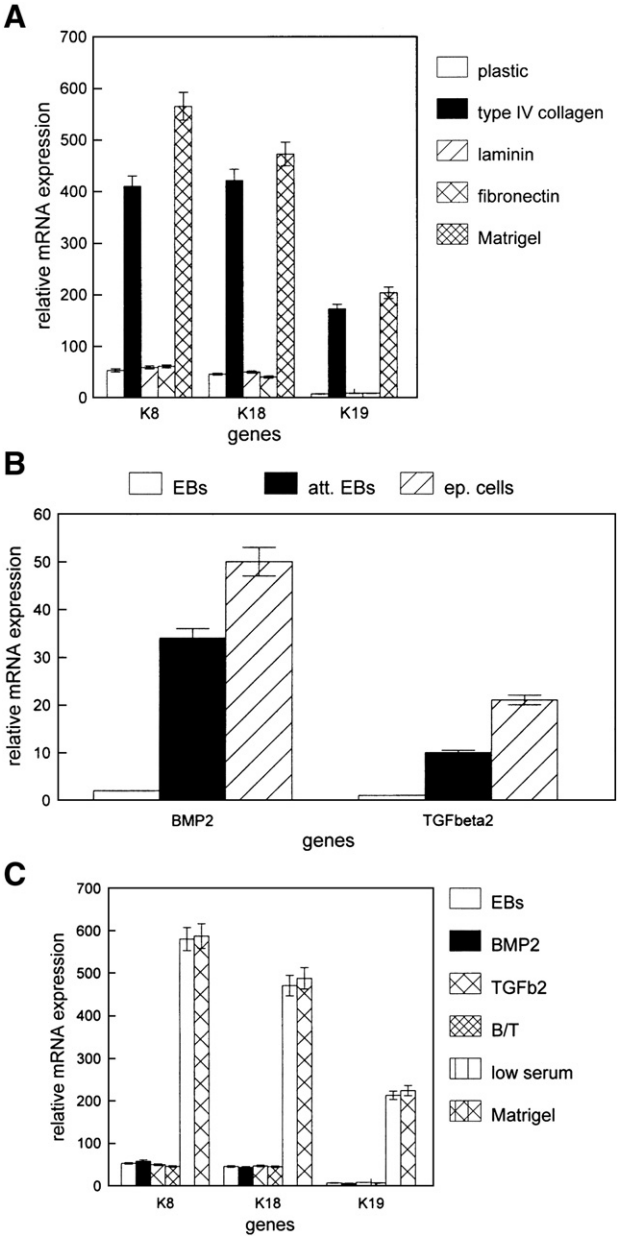


Figure 3 EB attachment to Matrigel induces higher levels of keratin expression than attachment to individual basement membrane components. (A) EBs were plated on plastic, type IV collagen, laminin, fibronectin, or Matrigel for 24 h prior to harvesting for RNA extraction. Keratin 8, keratin 18, and keratin 19 expression was determined by real-time RT-PCR. Error bars indicate SEM of three independent experiments. (B) Induction of BMP2 and TGFβ2mRNA expression during differentiation of EBs, basement membrane attached EBs (att. EBs), and the epithelial outgrowth of attached EBs (ep. cells). Real-time RT-PCR analysis was performed as described under Materials and methods. Error bars indicate SEM of three independent experiments. (C) Low serum but not growth factors induce EB epithelial differentiation in the absence of attachment to Matrigel. EBs cultured in suspension were treated with vehicle, BMP2, or TGFβ2 alone or in combination (B/T) or were subjected to low serum (2%) for 24 h as described under Materials and methods. Keratin expression in these groups was compared to EBs attached to Matrigel for 24 h. Keratin 8, keratin 18, and keratin 19 expression was determined by real-time RT-PCR. Error bars indicate SEM of three independent experiments.

Discussion

Previous studies proposed that migration of EB cells onto reconstituted basement membrane was required for

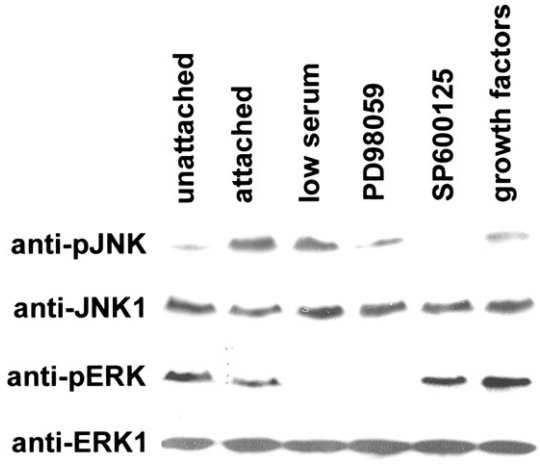


Figure 4 Increased ratio of JNK1/ERK1 activity correlates with epithelial differentiation of EBs. EBs were attached to Matrigel and treated with PD98059, SP600125, or growth factors for 24 h. Other EBs were grown in suspension with 15 or 2% serum for 24 h. Cells were harvested for protein extraction and subjected to Western blotting using activated JNK1 and ERK1 antibodies (pJNK1, pERK1) and total JNK1 and ERK1 antibodies. These experiments were performed three times with similar results. Representative blots are shown.

epithelial cell differentiation (Turksen & Troy, 1998; Troy & Turksen, 2006; Troy & Turksen, 2005). Our study examines mechanisms of epithelial differentiation in this system. Our data indicate that constituent cells of EBs attached to reconstituted basement membrane express epithelial-specific markers, not just those cells which migrate out of the EB onto Matrigel. This conclusion is supported by multiple lines of experimental evidence, including the genetic relatedness of the attached EBs to their epithelial progeny and weighs against epithelial differentiation occurring after cellular migration onto reconstituted basement membrane. These results suggest the existence of rapid signaling mechanisms in constituent cells adjacent to basement membrane. We determined that JNK1 was activated while ERK1 activity was reduced in EBs by attachment to Matrigel. Culture of EBs in reduced serum concentration also induced expression of JNK1 and epithelial differentiation markers. One possible explanation could be coordinate regulation of epithelial differentiation genes by reduced serum concentration via the JNK1 pathway. We determined that JNK signaling was critical for epithelial differentiation, since inhibition of this kinase blocked formation of epithelial cells. The JNK pathway is under negative feedback control of NF-κB and can result in apoptosis of cells (Kriehuber et al., 2005); NF-KB inhibition resulted in apoptosis of EBs in our culture system as did that of PKC signaling (Quinlan et al., 2003). Previous studies have shown that MAP kinase signaling is required for neural stem cell maintenance (Campos et al., 2004) and MEK inhibition promoted the growth of undifferentiated ES cells (Burdon et al., 1999). PI3K inhibition has been shown to decrease ES self-renewal and induce differentiated morphology (Paling et al., 2004).

We initially hypothesized that paracrine growth factor signaling would be a likely candidate mechanism, given that the epithelial-specific gene expression changes correlated with significant inductions of BMP and TGFβ expression. Our results indicate that these factors are not sufficient for development of stratified epithelium. During early embryonic development, BMP4 signaling is required for gastrulation and mesoderm formation (Winnier et al., 1995) and BMP2 null embryos have reduced numbers of primordial germ cells (Loebel et al., 2003). Interestingly, BMP signaling has been reported to both inhibit and induce ES cell differentiation (Ying et al., 2003; Pera et al., 2004). Inhibition of BMP signaling blocks expression of endodermal markers and cavitation of embryoid bodies (Coucouvanis & Martin, 1999) while activated BMP2 receptor induces epidermis formation in Xenopus ectodermal cells (Suzuki et al., 1997). TGFβ2 is expressed in adult epidermis (Wataya-Kaneda et al., 1994) and may have a role in maintaining the stem cell niche in skin (Tumbar et al., 2004), and downstream Smad2 signaling is required for endoderm formation in the early mouse embryo (Tremblay et al., 1999). Given that TGFβ ligands are downregulated in skin cancers (Glick et al., 1993), it is interesting to speculate that in the absence of TGFβ the adult stem cells in this tissue are no longer maintained in their niche, thereby contributing to tumorigenesis.

IGF2 has been shown to increase proliferation of the basal layer of epidermis, and imprinting of this gene is frequently lost in squamous cell carcinomas (El-Naggar et al., 1999; Bennett et al., 2003). We also noted in our microarray data that FGF4 was significantly downregulated in attached EBs and stratified squamous epithelial cells. Since FGF4 is sufficient to induce endoderm formation in germ layer explants (Wells & Melton, 2000), it is possible that stratified epithelial differentiation may require downregulation of FGF4 expression in order to inhibit endoderm formation. In separate experiments we treated EBs attached to reconstituted basement membrane with 10 ng/ml FGF4 to determine if this factor inhibited epithelial differentiation in this system. However, FGF4 treatment did not affect stratified squamous epithelial differentiation in basement membrane-attached EBs (data not shown). These results suggest that basement membrane attachment is sufficient to override the inductive effects of FGF4 on endoderm formation.

Additional regulators of stratified epithelial lineage commitment and differentiation during embryonic development remain to be determined. Integrin-mediated EB attachment was shown to be necessary for epithelial differentiation since β1 integrin null ES cells failed to produce keratinocyte markers when attached to gelatinized plates (Bagutti et al., 1996). Progressive onset of keratin expression was shown during the time course of the earlier study with wild-type ES cells producing terminal differentiation mRNAs (keratin 10, involucrin), but the corresponding proteins were not detected. Integrin β1 null ES cells also failed to undergo epithelial differentiation on deepidermized human dermis, but did express keratin 14 when cocultured with human dermal fibroblasts or conditioned media (Bagutti et al., 2001). TGFα and KGF, known regulators of keratinocyte proliferation, were shown to induce keratin 14 expression in this model. A separate study showed that human ES cells grown in nude mice expressed keratin 14 and involucrin proteins when cultured in vitro with feeder layers (Troy & Turksen, 2005). Our results indicate that EBs attached to reconstituted basement membrane undergo early commitment to epithelial differentiation

and when these cells migrate onto Matrigel they exhibit multiple features of stratified epithelium. These features include morphologic evidence of differentiation in culture and expression of relevant genes. The basement membrane component type IV collagen was best able to support simple epithelial differentiation. This result should be interpreted cautiously given that expression of type IV collagen and laminin is upregulated in the differentiating epithelial cells, likely resulting in additional reconstitution of basement membrane during the course of the experiment.

In a previous report (Conley et al., 2004), we noted some similarities in gene expression between stratified epithelial cells derived from embryonic stem cells and visceral endoderm. There are several key differences between these studies. In the previous report visceral endoderm formation was induced by long-term suspension culture (Conley et al., 2004), while our model used attachment to reconstituted basement membrane to induce stratified epithelium formation as independently demonstrated by other laboratories (Turksen & Troy, 1998; Troy & Turksen, 2006; Troy & Turksen, 2005; Green et al., 2003; Aberdam et al., 2008). Indeed Matrigel attachment has been shown to inhibit visceral endoderm formation (Rust et al., 2006). Alpha-fetoprotein, considered a visceral endoderm marker, also is expressed in developing epidermal cells (Trojan & Uriel, 1982). While the pan-keratin antibody used in the previous study recognizes both simple and stratified epithelial keratins, we demonstrated specific stratified epithelial keratin expression by RT-PCR, immunofluorescence, and Western blot. Additionally the endodermal marker GATA6 was not expressed by stratified epithelial cells in our model. Inhibition of ERK and p38 kinases has been shown to block endoderm formation (Liu et al., 2009), but had no effect on stratified epithelial cell formation in our model. Indeed JNK activity was required for stratified epithelial cell formation as demonstrated in Fig. 5. Visceral endoderm cells contain lipid droplets and robust microtubule networks visible by phase-contrast microscopy (Talbot et al., 2005) which were not observed in our stratified epithelial cells (Fig. 5). These results indicate that stratified epithelial cells derived from embryonic stem cells are morphologically and genetically distinct from visceral endoderm.

A previous study examined differentiation of EBs attached to gelatin or Matrigel (Sepulveda et al., 2008). This study proposed that bone-related genes were upregulated by gelatin and Matrigel attachment. However, a number of these genes were expressed in undifferentiated EBs, and expression of the osteoblast transcription factor Runx2 was not reported. Interestingly this study reported expression of keratins 8, 18, and 19 in agreement with our report. However

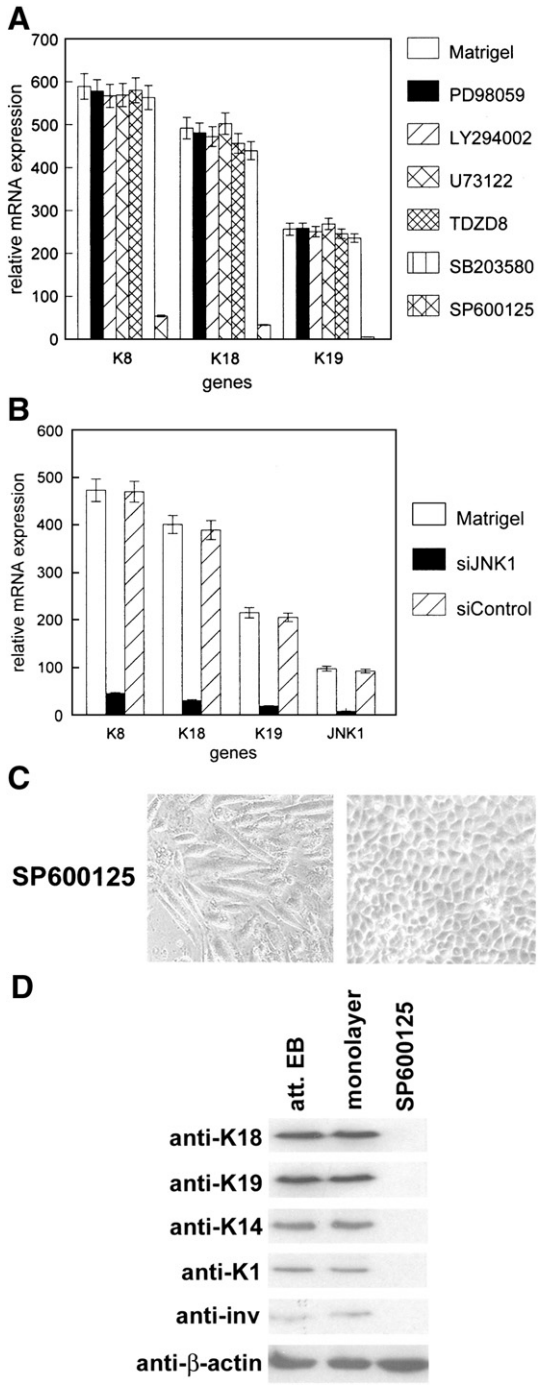


Figure 5 JNK1 inhibition is sufficient to block epithelial differentiation of EBs attached to Matrigel. (A) EBs attached to Matrigel were treated with vehicle, the MEK inhibitor PD98059, the PI3K inhibitor LY294002, the PLCγ inhibitor U73122, the GSK inhibitor TDZD8, the p38 kinase inhibitor SB203580, and the JNK1 inhibitor SP600125. Keratin 8, keratin 18, and keratin 19 expression was determined by real-time RT-PCR. Error bars indicate SEM of three independent experiments. (B) Inhibition of JNK1 expression blocks Matrigel-induced epithelial differentiation of ES cells. ES cells were transfected with siRNA to JNK1 (siJNK1) or control siRNA (siControl) prior to plating on Matrigel. Keratin and JNK1 expression was determined by real-time RT-PCR. Error bars indicate SEM of three independent experiments. (C) Morphologic appearance of EB outgrowth cells when attached EBs were treated with the JNK1 inhibitor SP600125 or vehicle. Note fibroblastoid morphology of SP600125-treated cells compared to typical cobblestone appearance of stratified epithelial cells in culture. (D) Expression of K18, K19, K5, K1, and filaggrin in attached EBs, monolayer epithelial outgrowth cells, and monolayers treated with the JNK inhibitor SP600125 was determined by Western blot. Experiments were performed three times with similar results. Representative blots are shown.

mechanisms leading to these changes were not proposed. We demonstrated that JNK signaling was required for epithelial differentiation of EBs to epithelial cells. While our paper was in review, a separate study reported that JNK signaling was required for proper differentiation of primordial germ layers from EBs (Xu & Davis, 2010).

EB attachment to reconstituted basement membrane strongly induces patterns of epithelial gene expression in constituent cells. Expression of a number of ion channel proteins, transporters, and kinases is upregulated during this process, providing attractive candidate mechanisms for future studies. It will be very interesting to test the abilities of these cells in regenerating epithelial tissues and their transformation potential (Aberdam, 2008). Ultimately the knowledge gained in these experiments combined with translational studies may have important therapeutic applications to a variety of human diseases.

Materials and methods

Cell culture

Mouse embryonic stem cells were derived from the blastocyst inner cell mass of the 129 mouse strain. Undifferentiated ES cells were cultured on STO mouse embryo fibroblasts that had been pretreated with 10 μg/ml mitomycin C for 3 h. ES cells were cultured in Dulbecco's modified Eagle medium (DMEM), 15% ES cell tested fetal bovine serum (FBS), 40 μg/ml gentamicin, and 1000 U/ml leukemia inhibitory factor. To induce differentiation, cells were dissociated with trypsin and cultured in suspension in DMEM, 15% FBS, and 40 μg/ml gentamicin for 7 days. The resultant embryoid bodies were plated on tissue culture dishes coated with reconstituted basement membrane (Matrigel) to allow outgrowth of differentiated cell types. EBs plated on uncoated tissue culture dishes were used as negative controls. Cultures were maintained for up to 14 days. Some EBs were cultured on dishes coated with basement membrane components (type IV collagen, laminin, fibronectin; Becton Dickinson). Other EBs were cultured in suspension with 6 ng/ml BMP2 and 2 ng/ml TGFβ2 alone or in combination. Control cultures were treated with bovine serum albumin vehicle solution for the same time periods. Cultures of EBs attached to Matrigel were treated individually with 10 μM PD98059 (MEK inhibitor; Alexis Biochemicals), 5 μM SP600125 (JNK inhibitor; Calbiochem), 5 μM SB203580 (p38 inhibitor), 10 μM SH5 (Akt inhibitor), 1 μM Go6976 (PKC inhibitor), 10 μM LY294002 (P13K inhibitor), 1 μM U73122 (PLCγ inhibitor), 10 ng/ml ras inhibitory peptide, 10 μM KN-62 (CaM kinase inhibitor), 10 μM TDZD8 (GSK inhibitor), or 10 μM panepoxydone (NF-κB inhibitor) for up to 6 days to determine which intracellular signaling pathways were involved in epithelial differentiation. To inhibit JNK1 gene expression, ES cells were transfected with siRNA to mouse JNK1 according to the manufacturer's recommendations (Dharmacon smartPool, No. 040128) prior to plating on Matrigel. Additional ES cell cultures were transfected with a control siRNA. To induce differentiation in the absence of basement membrane attachment, some EBs were cultured in 2% FBS. Cultures were photographed using phase-contrast microscopy.

Immunocytochemistry and Immunofluorescence

To determine if epithelial-specific gene products were expressed by cultured EBs, EBs plated on Matrigel from 6 h to 3 days were washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol for 20 min at 4 °C. After washing in PBS, the fixed cells were blocked with 10% normal serum followed by incubation with anti-keratin antibodies or control IgG for 1 h at room temperature. After washing in PBS, the cells were incubated with anti-mouse IgG secondary antibody conjugated to biotin or fluorescein for 10 min at room temperature. After additional washing in PBS, the cultures incubated with biotinylated secondary antibody were treated with streptavidin-conjugated horseradish peroxidase enzyme for 10 min at room temperature. Following final washes in PBS, antigen–antibody complexes were detected by incubation with hydrogen peroxide substrate solution containing aminoethylcarbazole chromogen reagent or by immunofluorescence following DAPI counterstain. Cultures were photographed using light or fluorescence microscopy.

Fluorescence-activated cell sorting (FACS)

To determine the percentage of epithelial cells in cultured EBs, EBs attached to reconstituted basement membrane were individually detached with a micropipette and aspirated. Single cell suspensions were created by trypsinization and following permeabilization with 70% ethanol were incubated with mouse anti-cytokeratin, filaggrin, or control IgG antibodies for 1 h at 4 °C. After washing in PBS, anti-mouse IgG secondary antibody conjugated to fluorescein was incubated with the cells for 2 h at 4 °C. After final washes, the fluorescent epithelial cell fraction was obtained by FACS using a Becton Dickinson FACSvantage dual laser/four color flow cytometer.

RNA extraction and gene expression profiling

Total RNA was extracted from all groups of cells using a commercially available kit (RNEasy, Qiagen, Valencia, CA). Integrity of ribosomal RNA bands was confirmed by Northern gel electrophoresis. Total RNA (10 μg) was converted to labeled cRNA targets. The biotinylated cRNA targets were then purified, fragmented, and hybridized to GeneChip mouse genome 430 2.0 expression arrays (Affymetrix, Santa Clara, CA) to interrogate the abundance of 39,000 possible transcripts in each sample. Affymetrix GCOS software was used to generate raw gene expression scores and normalized to the relative hybridization signal from each experiment. All gene expression scores were set to a minimum value of 2 times the background determined by GCOS software in order to minimize noise associated with less robust measurements of rare transcripts. Normalized gene expression data were imported into dChip software (http://www.biostat.harvard.edu/complab/dchip) for hierarchical clustering analysis using the average linkage algorithm. Data were analyzed by *t* test with $P<0.005$ followed by ratio analysis (minimum 2-fold change).

RT-PCR

Embryoid bodies were individually detached from monolayer epithelial cells with a micropipette tip followed by aspiration. RNA was extracted from all cells using a commercially available kit (Qiagen) and reverse-transcribed using SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA was amplified using the following mouse primers:

BMP2: 5′-TGTGAGGATTAGCAGGTCTTTGC-3′ and 5′-GTTAGTGGAGTTCAGGTGGTCAGC-3′

TGFβ2: 5′-ACACACCAAAGTCCTCAGCCTG-3′ and 5′-TCCCTCCCTCCTGTCACAAACATC-3′

JNK1: 5′-AGGTGCAGCAATGATCAATG-3′ and 5′-ATCAATGACTAACCAATTCCCC-3′

Keratin 8: 5′-AAACCCGAGATGGGAAGCTG-3′ and 5′-GTTTGGATGGGAGGCAGAAG-3′

Keratin 18: 5′-AGGCAGAGATTGCCACCTAC-3′ and 5′-TTTTATTGGTCCCTCAGTTCCC-3′

Keratin 19: 5′-AGAACCAGGAGTATAAGCAGC-3′ and 5′-ATTGACAAGTCGAGGGAGG-3′

Keratin 5: 5′-TTCTTTTAGGCAGTCCCCC-3′ and 5′-GGTGGCTTACATTCTGCAAC-3′

Keratin 14: 5′-ACCAAGGTCATGGATGTGC-3′ and 5′-GCTTTATTGCTTGCCAGGAAG-3′

Keratin 1: 5′-TCAGTTCATCACCAACATGAC-3′ and 5′-GCAGCAAAACAAAGAAACGG-3′

Keratin 10: 5′-TGACACCTGAGGGTAGAGTC-3′ and 5′-ACCACAAAACACCTTTTAGACC-3′

Involucrin: 5′-AAATCTATCTGGCCCTGACC-3′ and 5′-CACAATTCTGCCACAACATTAG-3′

Filaggrin: 5′-AATGCTTAAATGCATCTCCAGG-3′ and 5′-GCTGAAGAAAGGGCAGATCC-3′.

The iScript One Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) was used in these experiments. Amplification with β-actin cDNA using primers 5′-AAGGCGACAGCAGTTGGTTG-3′ and 5′-TGGGGGGACAAAAAAAAGGG-3′ as the internal control was carried out by real-time PCR (iCycler iQ, Bio-Rad) using cycle parameters of 94 °C for 25 s, 55 °C for 1 min, and 72 °C for 1 min. Data were statistically analyzed by ANOVA.

Western blot

The amount of 75 μg total cellular proteins from growth factor-treated EBs and control cells was separated by SDS-PAGE on 10% resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted to PVDF membranes according to the manufacturer's recommendations (Roche Molecular Biochemicals). Blots were incubated with antibodies to activated and total JNK1 and ERK1 proteins (Santa Cruz Biotechnology) overnight at 4 °C. After washing in Tris-buffered saline containing 0.1% Tween 20 (TBST, pH 7.4), blots were incubated for 30 min at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals). Bands were quantitated by densitometry.

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